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# CONVERSION OF ORNITHINE INTO PROLINE BY ENZYMES FROM GERMINATING PEANUT COTYLEDONS

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**Abstract**—During germination a marked synthesis of proline occurs in the cotyledons of peanuts. A soluble enzyme system which converted ornithine into proline was extracted from acetone powders of 3–5-day-old peanut cotyledons. The reaction required ornithine, α-ketoglutarate, and a reduced pyridine nucleotide. NADPH was much more effect than NADH. An L-ornithine:2-oxoacid aminotransferase (EC 2.6.1.13) has been purified about six-fold from the extracts. The reaction produces a pyrroline carboxylate as indicated by a positive reaction with *o*-aminobenzaldehyde. An optimum pH of 8·0 was found with tris-HCl. The $K_m$ for α-ketoglutarate was 2·5 mM and for L-ornithine 5·0 mM. Exogenous pyridoxal phosphate was not required, but 0·1 mM hydroxylamine inhibited the reaction by 90 per cent. α-Ketoglutarate was quite specific as the acceptor, pyruvate, oxaloacetate, and glyoxylate showing very little activity. Fractionation of germinating cotyledon homogenates showed that the mitochondrial fraction had a specific activity more than eight-fold that in the remaining soluble cytoplasm, but the total soluble activity was six times that of the mitochondrial fraction.

## INTRODUCTION

THE ROUTE of biogenesis of proline from glutamic acid or ornithine has been established in microorganisms and mammals.[1] One of the intermediate steps is the formation of glutamic-γ-semialdehyde.[2,3] Enzymes catalyzing the δ-transamination of ornithine to glutamic-γ-semialdehyde have been purified from rat liver,[4,5] *Neurospora crassa*,[6] *Chlamydomonas reinhardi*,[7] and *Tetrahymena pyriformis*.[8] Glutamic-γ-semialdehyde exists in equilibrium with its cyclic form $\Delta^1$-pyrroline-5-carboxylate. The reduction of this compound to proline has been accomplished using enzymes from rat tissues,[9,10] calf liver,[11] and various microorganisms.[8,9]

A similar pathway for the formation of proline is apparently present in higher plants

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although the evidence is much less detailed. After feeding ornithine-$^{14}$C to seedlings of several species, good incorporation of the label into proline has been observed.[12, 13] The reduction of glutamic acid-$^{14}$C to labelled proline has been followed in some detail in feeding experiments with whole tobacco leaves[14] and leaf discs.[15] Transaminases which utilize L-ornithine as a substrate have been detected in cell-free preparations from seedlings of green pea,[16] wheat,[16] mung bean,[17] and sunflower.[18] In the latter two instances the enzyme was apparently localized in the mitochondria. The mung bean mitochondrial enzyme has been solubilized and shown to form glutamic-$\gamma$-semialdehyde from ornithine.[19] A $\Delta^1$-pyrroline-5-carboxylate reductase has recently been partially purified from tobacco leaves.[20]

In the course of germination of peanut (*Arachis hypogaea*) seedlings, a large increase was observed in the amount of proline in the free amino acid pool. After 3 days at 30° in the dark, proline was the dominant constituent based on ninhydrin-treated paper chromatograms. Extracts of acetone powders of the cotyledons contained an enzyme system which oxidized NADPH or NADH in the presence of L-ornithine and $\alpha$-ketoglutarate. Proline was obtained as a product. The demonstration of the overall conversion of ornithine into proline and the partial purification and properties of an L-ornithine:2-oxoacid aminotransferase (EC 2.6.1.13) from these extracts are presented in this report.

## RESULTS

### *Spectrophotometric and Chromatographic Evidence for Overall Conversion*

In the presence of enzymes required for the overall conversion of ornithine into proline, the oxidation of reduced pyridine nucleotides should be dependent on the presence of ornithine and $\alpha$-ketoglutarate. Figure 1 shows that the oxidation of added reduced pyridine nucleotides by a dialyzed ammonium sulfate fraction prepared from an extract of an acetone powder of germinated peanut cotyledons required both of the above compounds. NADPH appeared to be more effective as a reductant than NADH when each was used independently. Analysis of the various reaction mixtures by paper chromatography established that in the absence of the reduced coenzyme, glutamic acid was formed and another ninhydrin-reactive material, but no proline was detected until either NADH or NADPH was added. In the presence of the reduced coenzyme proline was easily detected on paper chromatograms after treatment with ninhydrin.

### *Partial Purification of Ornithine Transaminase*

Ten grams of an acetone powder of cotyledons from 3-day-old peanut seedlings were extracted with 100 ml of cold 0·2 M potassium phosphate buffer, pH 7·2. All succeeding operations were carried out in the cold (4–10°). After extraction for 10 min the mixture was strained through several layers of muslin and the filtrate centrifuged for 30 min at 23,000 *g*. The supernatant solution was decanted and 0·15 ml of a 1% protamine sulfate solution was

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added with stirring for each milliliter of supernatant fluid. After 10 min stirring the mixture was centrifuged and the precipitate discarded. Solid $(NH_4)_2SO_4$ was added to the solution until 35% saturation was reached (243 mg per ml). The mixture was left for 10 min and the precipitate removed by centrifugation and discarded. The supernatant was made to 55% saturation with $(NH_4)_2SO_4$ by the further addition of 97 mg per ml. The precipitate was

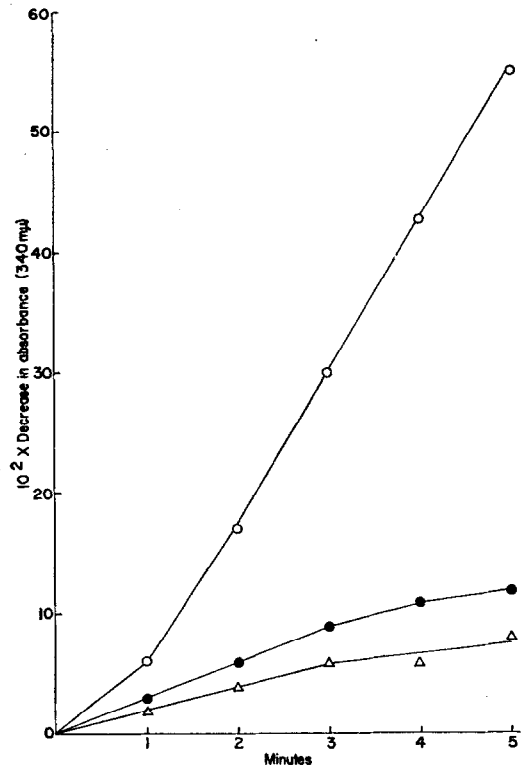


FIG. 1. CONVERSION OF ORNITHINE TO PROLINE MEASURED BY OXIDATION OF REDUCED PYRIDINE NUCLEOTIDES.

Enzyme was prepared from an acetone powder extract by making extract to 75% saturation with $(NH_4)_2SO_4$ and dissolving precipitate in 0·2 M potassium phosphate buffer, pH 7·4, and dialysing against 0·05 M phosphate buffer at pH 7·4 overnight. The dialysed solution was used as the enzyme source. The complete reaction mixture contained in a final volume of 3 ml, the following components: Tris-HCl pH 8·2, 0·17 M; NADPH, 0·13 mM; NADH, 0·13 mM; α-ketoglutarate, 3·3 mM; L-ornithine, 2 mM; pyridoxal-5′-phosphate, 33 μM; enzyme protein, 9·6 mg. Reaction carried out as described in "Methods".

○——○, complete; ●——●, minus ornithine; △——△, minus α-ketoglutarate

collected by centrifugation after 10 min and dissolved in 0·2 M phosphate buffer, pH 7·2. The summary of a typical purification is given in Table 1.

*Kinetic Parameters*

The pH optimum for transamination was found to be 8·0 using tris-HCl buffer. The optimum was well defined with the activity falling off more rapidly on the acid side.

Under the assay conditions, transamination occurring was proportional with time for at least 60 min and the reaction rate measured was linearly related to the amount of enzyme

used. The reaction showed Michaelis–Menten kinetics and the $K_m$ for each substrate was determined in the presence of saturating levels of the other substrate. Figure 2 shows the graphical determination of the constants and the effect of substrate concentration on the reaction rate. $K_m$ values calculated were 5·0 mM for L-ornithine and 2·5 mM for α-keto-glutarate.

TABLE 1. PURIFICATION PROCEDURE

| | Protein (mg) | Specific activity (milliunits/mg) | Purification | Recovery (%) |
|---|---|---|---|---|
| Fraction | | | | |
| I. Acetone powder extract | 1470 | 4·7 | 1·0 | 100 |
| II. Protamine sulfate supernate | 648 | 8·8 | 1·8 | 83 |
| III. 35–55% $(NH_4)_2 SO_4$ Fraction | 188 | 25·9 | 5·5 | 70 |

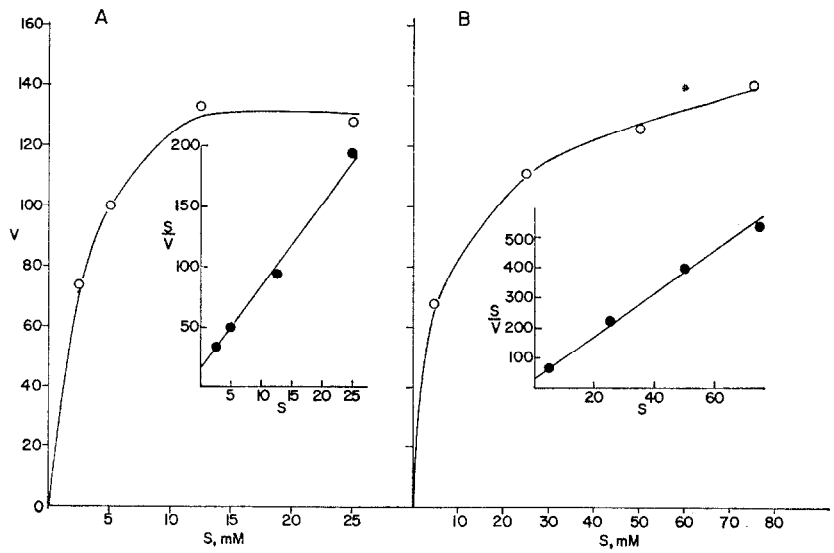


FIG. 2. TRANSAMINATION AS A FUNCTION OF SUBSTRATE CONCENTRATION.

A. Standard reaction mixture containing 2·5 mg protein (except for variation of α-ketoglutarate as indicated). Reaction time 10 min. B. Standard reaction mixture except for ornithine. Incubation time 10 min at room temperature. Fraction III protein (2·5 mg) used as enzyme source.

*Reaction Requirements*

The only requirements for the reaction were enzyme, L-ornithine, and α-ketoglutarate (Table 2). The addition of exogenous pyridoxal-5′-phosphate stimulated the reaction very slightly. Other keto acids could not replace α-ketoglutarate. Pyruvate and oxaloacetate only gave 4 per cent of the rate measured with α-ketoglutarate. Glyoxylate was somewhat more effective but still gave only 11 per cent of the activity of α-ketoglutarate.

TABLE 2. TRANSAMINASE REACTION REQUIREMENTS

| Reaction mixture | Pyrroline carboxylate formed (mμmoles) |
|---|---|
| Complete | 385 |
| Complete, minus ornithine | 0 |
| Complete, minus α-ketoglutarate | 37 |
| Complete, plus pyridoxal phosphate, 50 μM | 397 |
| Complete, using heat-inactivated enzyme | 59 |

The complete reaction mixture was that described in Methods. Enzyme used was 1·3 mg of Fraction III (see Table 1).

*Intracellular Distribution*

Cotyledons from 4-day-old peanuts were blended in the cold for 1 min at full speed in a Waring blendor with an equal volume of a solution containing 0·25 M sucrose and 0·05 M potassium phosphate, pH 7·2. The homogenate was strained through muslin and then centrifuged at 4° for 5 min at 1000 *g*. The supernatant fluid was decanted and centrifuged for 20 min at 20,000 *g* in the cold. The supernatant was decanted and used as the source for cytoplasmic enzymes. The precipitate was resuspended in fresh sucrose-phosphate solution and recentrifuged for 20 min at 20,000 *g*. The sediment was suspended in a small volume of cold sucrose-phosphate solution and portions used to determine the activity of the particulate components of the cell, in this case primarily the mitochondria and the broken mitochondrial fragments. The cytoplasmic fraction and the particulate suspension were tested for transaminase activity. The results are given in Table 3. The specific activity of the washed particles was over eight times that of the cytoplasm; however, the cytoplasm contained six-fold more total activity.

TABLE 3. INTRACELLULAR LOCALIZATION OF TRANSAMINASE

| | Specific activity (milliunits/mg protein) | Total activity (milliunits) |
|---|---|---|
| Washed particles | 23·8 | 5,210 |
| Cytoplasm | 2·8 | 30,200 |

*Effect of Various Amino Acids and Inhibitors*

The purified ornithine-δ-transaminase of rat liver was inhibited markedly by L-canavanine, δ-aminovalerate, and L-valine.[5] The enzyme from *Tetrahymena pyriformis* is also inhibited strongly by L-valine.[8] The effect of these amino acids, proline, and lysine on the peanut enzyme was examined. L-Canavanine, L-valine and DL-norvaline all produced substantial inhibition but the other amino acids tested had little or no effect at the concentrations used (Table 4).

Exogenous pyridoxal phosphate had little effect. In the presence of 0·1 mM hydroxylamine the enzyme activity was 90 per cent inhibited, which implies that a carbonyl function is required. If pyridoxal phosphate is involved it must be tightly bound to the enzyme. Other

common inhibitors or activators had little effect, EDTA and dithiothreitol being only slightly stimulatory. Pre-incubation with 1 mM iodoacetamide for 5 min had no effect on the enzyme's activity.

TABLE 4. INHIBITION BY VARIOUS AMINO ACIDS

| Amino acid | Concentration (mM) | Inhibition (%) |
|---|---|---|
| L-Canavanine | 30 | 42 |
| δ-Aminovalerate | 30 | 7 |
| L-Valine | 30 | 33 |
| DL-Norvaline | 60 | 31 |
| L-Proline | 10 | 0 |
| L-Lysine | 10 | 0 |

The reaction was carried out as described in "Methods". Fraction III protein (2 mg) was used as the source of enzyme.

*Age of Cotyledon and Transaminase Activity*

Acetone powders of cotyledons of peanuts after 0, 1, 2, 3, and 6 days of germination were prepared. Extracts were made as described previously using phosphate buffer and then centrifuged at 23,000 *g* for 30 min. The supernatant fraction was assayed for transaminase activity. The specific activities obtained were 0·8, 2·4, 2·7, 4·2, and 5·8 respectively.

*Transaminase in Other Species*

Ornithine transaminase has been reported in a number of other plant species.[16–19] The acetone powder procedure was tested with 2-day-old mung bean cotyledons, leaves of *Polygonatum multiflorum*, mature marrow fruits, and cauliflower buds. Extracts of each tissue were clarified by centrifugation as above before use. The specific activities obtained were 1·75, 1·75, 14·1, 0·9 respectively.

*Reduction to Proline*

In Fig. 1 the overall conversion of ornithine to proline was demonstrated and shown to require reduced pyridine nucleotide, α-ketoglutarate, and ornithine. The most purified fraction obtained for the transaminase was found capable of catalyzing the final reduction to proline. It was necessary to remove $(NH_4)_2SO_4$ remaining after the fractionation procedure by dialysis, otherwise contaminating glutamic dehydrogenase interfered with the measurement of the activity of the pyrroline reductase. Controls in which ornithine was omitted were used to determine residual glutamic dehydrogenase activity and to correct for the oxidation of NADPH or NADH by this enzyme.

The rate of oxidation of NADPH was much higher than that of NADH with this enzyme fraction. An interesting aspect of this differential utilization was the effect of storage of the enzyme at −10°. After 7 days the rate of the reaction with NADPH had decreased about 12–20 per cent but the rate with NADH had diminished by 75 per cent. These results are summarized in Fig. 3.

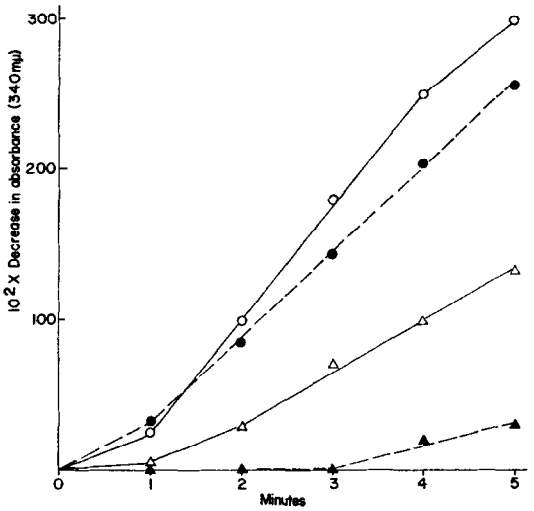


FIG. 3. EFFECT OF STORAGE ON PYRIDINE NUCLEOTIDE SPECIFICITY OF PYRROLINE REDUCTASE.
Standard reaction mixture utilizing Fraction III protein (4·6 mg) dialysed for 4 hr against 0·05 M potassium acetate, pH 5·5. The precipitate formed during dialysis was discarded. The curves are corrected for the oxidation of NADPH or NADH in the absence of ornithine.
○, ● NADPH, △, ▲ NADH, —— original activity, ----- after 7 days at −10°.

## DISCUSSION

The results of the present study allow the comparison of a higher plant ornithine transaminase with a number of similar enzymes from different organisms. The Michaelis constants for α-ketoglutarate and ornithine were quite similar regardless of source, except for the results of Strecker[5] with a purified rat-liver enzyme. The *Neurospora* enzyme[6] and rat-liver enzyme[4,5] had pH optima at 7·1–7·4. The *Chlamydomonas reinhardi*[7] and *Tetrahymena pyriformis*[8] enzymes had their optima at pH 8·0 and 9·0 respectively, which is more in accord with the peanut enzyme. The *C. reinhardi* enzyme in fact had a $K_m$ for L-ornithine of 4·45 mM and for α-ketoglutarate of 2·55 mM, values remarkably similar to those found for the peanut transaminase. The major difference between the algal enzyme and the peanut enzyme lay in the ease of removal of bound pyridoxal phosphate from the former, and in the marked inhibition of the algal enzyme by a number of amino acids particularly valine and norvaline. The rat-liver enzyme was strongly inhibited by L-canavanine and δ-aminovalerate.[5] The peanut enzyme was inhibited to some extent by the above amino acids when used at quite high concentrations with the exception of δ-aminovalerate which showed very little inhibitory effect.

As with ornithine transaminases from other tissues, the enzyme of peanut cotyledons was present in the mitochrondria and mitochondrial fragments in high concentration. There was, however, very much more total activity in the cytoplasmic fraction of the cell. The possibility is not eliminated that there may be two enzymes, one soluble and the other particulate. A recent report by Hasse *et al.*[21] describes transaminases extracted from *Lupinus angustifolia* and *Phaseolus aureus* which deaminate ornithine at the α-position. The *Lupinus* enzyme has been purified to some extent and has an optimum pH of 6·0.

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Our results have shown a steady increase in the specific activity of the enzyme from the peanut cotyledons with the age of the cotyledon. The greatest increase takes place immediately upon germination, but the increase continues up to the sixth day of germination. This may not reflect actual synthesis of enzyme molecules but rather the preferential utilization of storage protein which effectively increases the relative concentration of transaminase.

The final reduction to proline used NADPH and NADH. The results in Fig. 2 show that on storage the NADH activity decreases very rapidly, whereas the NADPH activity remains quite high. This strongly indicates that possibly two enzymes are involved. It has often been suggested that synthetic enzymes prefer NADPH and enzymes concerned with degradation NADH. Thus the NADH activity could reflect the activity of a proline dehydrogenase. We have recently observed such an activity in our extracts which utilizes NAD much more effectively than NADP.[22]

## MATERIALS AND METHODS

### *Chemicals*

Amino acids and other compounds were obtained commercially. The $(NH_4)_2SO_4$ used was especially low in heavy metals. *o*-Aminobenzaldehyde was purchased from Sigma Chemical Co. and also synthesized by the method of Albrecht *et al.*[23]

### *Preparation of Acetone Powders*

Peanuts, a Mexican variety, were purchased from a local dealer. Seeds were dusted with the fungicide, thiram (50% tetramethyl thiuram disulphide), and germinated in moistened vermiculite in the dark at 30°. The cotyledons were harvested when 3–5 days old. They were washed in distilled water and any necrotic areas were excised before blending in 4 volumes of acetone (at −15°) for 1 min at full speed. The homogenate was filtered by suction and the precipitate dried in air at room temperature until free of acetone. The powder was stored at 10° until used.

### *Assay Procedures*

The standard reaction mixture for assay of transaminase activity contained the following materials in a volume of 2 ml:

| | |
|---|---|
| Tris-HCl buffer, pH 8·0 | 100 mM |
| α-Ketoglutarate, dipotassium salt with KOH | 12·5 mM |
| L-Ornithine | 50 mM |
| Enzyme | |

The mixture was incubated with occasional shaking at room temperature for 15 min, and then the reaction was terminated and assayed for the pyrroline carboxylate product by the method of Johnson and Strecker.[24] The blank contained all the components except enzyme. The amount of pyrroline carboxylate formed was calculated using a millimolar extinction coefficient of 2·71. A unit of enzyme activity is defined as that amount of enzyme which converts 1 μmole of substrate per min at 23°.

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When the overall conversion of ornithine into proline was measured by oxidation of reduced pyridine nucleotides at 340 nm, the complete reaction mixture consisted of the following in a final volume of 3 ml, except in the case of Fig. 1:

| | |
|---|---|
| Tris-HCl buffer, pH 8·0 | 100 mM |
| α-Ketoglutarate, dipotassium salt | 12·5 mM |
| L-Ornithine | 50 mM |
| NADPH or NADH | 0·13 mM |
| Enzyme | |

The reaction was started by the addition of enzyme or pyridine nucleotide, and the rate followed by use of a recording spectrophotometer. Protein was estimated by the biuret method.[25]

*Acknowledgement*—This study was carried out during the tenure of one of the authors (M. M.) as an honorary research associate in the Department of Botany, University College London.

[25] E. LAYNE, in *Methods of Enzymology* (edited by S. P. COLOWICK and N. O. KAPLAN), Vol. 3, p. 450, Academic Press, New York (1957).